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(57) Abstract : **The inv nti n concerns novel uses f male sterility to improve the cultivation conditions of transgenic plants f r man and environment.**

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New uses of male sterility among the plants

The invention concerns new uses of male sterility in order to limit your risks linked to the production of transgenetic plants for man and the environment.

The advantages of the use of transgenetic plants are very promising. The transgenesis of genes of mammals in a vegetable cell in particular offers a line of production in large quantities of new recombinant proteins, against a reduced production cost and without the risk of viral infections or subviral infections (we hope).

However, these advantages should not make us forget the aspects of an ecological nature linked to the production of transgenetic plants, for which the public reacts in a very sensitive way.

The Plaintiff presently finalized a technology putting male sterility at the disposal of a "green biotechnology", respectful for the ecological and human aspects.

A possible improvement lies in the control of the dissemination of the transgene in the environment.

The spatial insulation of the cultivated fragments already allows you to reduce the risk by "leaks" of the transgene, but this method is difficult and becomes a problem with the multiplication of the products.

The advantage of genetic methods in order to isolate the transgene and in order to reduce the risk by "leak" has been suggested in an article of Ellstrand in 1990. Among numerous proposals of solutions with regard to the problem of
"leak" of the transgene, Ellstrand observes that the male sterile genotypes could be introduced, or that a lethal gene for the pollen could be directly linked to a gene built by a genetic genius. However, in this sense no technical development had been implemented. It was impossible to predict the success of the implementation of such development, especially because of the number of parameters involved in the techniques of the genetic genius among the plants.

The Plaintiff finalized a technology allowing to prevent the dissemination of the transgene by means of a pollen line and therefore its "leak" in the environment; it is a technology according to which a sterile male plant is used in order to avoid the dissemination of an advantageous transgene integrated into the genome of the said plant.

The advantage of the control of your male fertility is known for the production of hybrid plants that involve the junction of two different lines.

One of the methods in order to prevent the self - fertilization by means of controlling the pollination is called the manual "castration" of the male organs of the plant.

Research is also done for a genetic control of the development of pollen.

Male sterility can be "obtained", that is it is independent of an ordinary genetic manipulation by means of the recombinant DNA. One can distinguish male cytoplasmic sterility of male nuclear sterility (Williams, 1995). Male cytoplasmic sterility is linked to changes in the organization and the expression of the mitochondrial genome, the male nuclear sterility is the result of changes in the genome of the nucleus of the cell.

The male sterility can also be "artificial", that is that it is induced by the expression of a gene resulting in male sterility (AMS gene), which is inserted either in the mitochondrial genome (cytoplasmic male sterility) or in the nuclear genome (nuclear male sterility).

In accordance with the invention, the said sterile plant used in order to avoid the dissemination of an advantageous transgene integrated into the genome of the said plant is bearer of :

- either of a cytoplasmic male sterility, preferably an artificial male sterility induced by a transgene integrated into the mitochondrial genome of the said plant ;

- or of a nuclear male sterility, preferably a male sterility induced by a transgene inserted in a non essential site of the nuclear genome of the said plant.

In a preferential way in accordance with the invention, the said plant is possibly made male sterile by means of the insertion in a non essential site of the nuclear genome, of a sequence containing an AMS gene and the said genetically advantageous transgene that is linked.

The connection between the advantageous gene and the AMS gene will result in the impediment of its dissemination by means of pollen, thus allowing a multitude of different productions in a same environment.

Moreover, the transfer of the AMS gene itself towards the plants in the wild should not be feared as it will not be susceptible to be held back within a wild population in so far as it no longer represents a "genetic load" besides the ordinary selective advantage.

By means of a connection between an advantageous gene and an AMS gene, a sufficiently small genetic distance is meant so that the frequencies of recombination during the meiosis are neglectable.

By means of genetic transformation, one can introduce in a plant two, indeed three genes by which the physical connection is absolute.

The invention also aims at a transgenetic plant, a part or an extract of a transgenetic plant, characterized by the fact that the said advantageous transgene is genetically linked with an AMS gene linked to elements allowing its expression in the vegetable cellules, especially a promoter and a transcription terminator.

In accordance with the present invention, it is clear that the presence of the said AMS gene does not prevent the expression of the said transgene of interest.

By "part" of transgenetic plant, we understand in particular leafs, fruits or cellules of genetically transformed plants.

The invention also aims at a vector, in particular plasmid, characterized by the fact that it contains a transgene of interest linked with elements allowing its expression in the vegetable cellules, in particular a promoter and a transcription terminator, genetically connected with an AMS gene linked with elements allowing its expression in the vegetable cellules, in particular a promoter and a transcription terminator.

According to a preferred production method of the invention, the artificial male sterility can be provided by a gene constituted of a sequence coding a protein susceptible to damage the cellular ARN (RNAse) under control of a specific anther promoter as is described by PAUL W. and al. (1992).

The RNase can be the Barnase of Bacillus amyloliquefaciens. The promoter can favorably be the A9 promoter of Arabidopsis thaliana specifically of the carpet of the anther.

The plants expressing this imaginary gene become incompetent to produce viable pollen thanks to the specific destruction of the cellules of the anther carpet. Such plants are otherwise normal.

With the plants according to the invention being incompetent to produce viable pollen, there is no risk of dissemination of the genes of male sterility and of interest because of the pollen.

The multiplication of these plants is done by means of introducing pollen deriving from plants that do not bear the gene of interest according to the invention.

In the case of corn, the cultivation of the transgenetic plants aiming at the production can be guaranteed according to a project of the type of production of seeds (4 or 6 female lines followed by 2 lines of pollinators). The plants used as females (plants bearing the gene of interest) are sowed in double density. In such project the advantageous gene is connected to a selected gene (for instance, a gene resisting a herbicide) which, consequently, allows, by means of a treatment with the selected agent(a herbicide for instance), to eliminate the plants other than the sterile males. Harvesting is only done on the female lines.

For the final production, a mixed semis can also be contemplated. In this case, the pollinator lineage is not bearing the advantageous gene and is used in mixture (10 %) with the lineage used as female. The semis is realized in double density. In this case the field is harvested wholly.

According to another method of production of the invention, male cytoplasmic sterility is used in order to limit the dissemination of a transgene integrated into the genome of a plant.

The plants bearing a male cytoplasmic sterility are affected in their mitochondrial genome, which makes them unfit for the production of pollen in absence of the nuclear restoration gene. This male sterility can either be "obtained" or artificially caused by the insertion of a gene imparting male cytoplasmic sterility (CMS gene) in the mitochondrial genome, for instance the Ogura gene.

Such plants are customarily used by the sowing factory in order to make their production of seeds easier. An important part of production of seeds of corn rests, for instance, in Europe with the use of the said male cytoplasmic sterility of type C.

According to a preferred method of production of the invention, a transgene is introduced by means of back - cross - fertilization in a plant's lineage, especially corn, bearer of male cytoplasmic sterility. The sterile male plant is in this case used as a female relative. Two successive cross - fertilizations followed by of evaluations and selections in the lineage allow you to obtain the homozygotic state for this transgene.

In absence of a nuclear restoration gene (gene Rf4 for the C cytoplasm), the bearer plant of the transgene will therefore again be made male sterile. Thus, no grain of bearer pollen of a transgene will be emitted in the environment. If the plant is homozygotic for the transgene, all the harvested grains are bearer of the gene and, therefore, express the phenotype.

The culture of these plants with a aim to produce, is realized by means of cultivation of the pollinating plants nearby male sterile transgenetic plants. This can be obtained either by means of mixing seeds (10 % of pollinator is sufficient) or by means of an alternation of female lines (sterile males) and pollinator lines. In the latter case, only the females are harvested.

The invention also aims at a production process of a product expressing a transgene of interest characterized by the following :

a) - either by the transformation of vegetable cellules, in particular with the help of a cellular host such as is previously defined, which itself is transformed by a vector containing a transgene of interest linked with elements allowing its expression in the vegetable cellules, genetically connected with an AMS gene linked with elements allowing its expression in the vegetable cellules, in that way to integrate an AMS gene, which is genetically connected with a transgene of interest, into the genome of these cellules:

- or by the transformation of vegetable cellules bearing a male cytoplasmic or nuclear sterility in such way to integrate a transgene of interest into the genome of these cellules ;

b) the regeneration of transformed plants from the above-mentioned transformed vegetable cellules,

c) the recuperation of the product expressing the said transgene of interest in the said cellules or the above-mentioned transformed plants, especially by means of extraction, followed, if necessary, by a purification.

Another aspect linked with the production of transgenetic plants lies in the presence of genes, possibly judges to be undesirable, or even pernicious, for man and/or the environment, in particular according to the public.

Indeed, it is necessary to sort out of among millions of cellules, those consisting of the modification to be introduced, in order to obtain transgenetic plants. In order to do so, genes of the markers that customarily impart resistance to antibiotics or herbicides are used. These gene are sometimes thought to be undesirable and different strategies (co-transformation, use of recombinants...) were considered in order to attempt to eliminate them.

Thus, the claim of claim WO 92 01370 divulges a process in order to produce a transgenetic plant containing an advantageous gene, freed of marker genes using a system of transposition.

Otherwise, the claim WO 91 09957 divulges a process of site recombination -specifically in the plants' cellules making use of a Cre/lox system in order to produce a deletion, an insertion or a mutual exchange of DNA segments. The described process is applicable to the elimination of a marker gene. The depositor also quotes the use of this process in order to restore fertility with regard to the production of hybrid seeds by a genetic genius.

However, in the former art the male sterility had not been used as a marker, such as in screening operations.

According to a method of production of the invention, the AMS gene can serve as positive marker for the screening of plants having integrated a transgene of interest, by which the individuals in possession of the said AMS gene are being selected.

The presence of this AMS gene can be detected especially by means of molecular analyses using polymerization chain reactions (PCR) and/or by means of the Southern blot according to the usual techniques (Sambrook and al., 1989). The plants having male sterility can also simply be selected by means of observing the presence or the absence of a development of grains of pollen. The AMS gene having served as a positive marker can then be regarded as undesirable and can be cut away.

According to another method of production of the invention, the AMS gene allows the improvement of the process of elimination of a fragment of undesirable exogenous DNA with regard to the screening operations of genetically transformed plants.

Indeed, in order to be effective, the existing strategies of screening, based on the use of a marker gene, for instance as described in the aforesaid claims, must work on a very high frequency. The cellules or plants having lost the marker gene are not to be selected anymore in comparison to those that have been stemmed. The sorting is then based on heavy and onerous molecular methods.

The Plaintiff presently discovered the advantage of the use of a gene imparting artificial male sterility (AMS gene) as "marker suicide" or "negative of an event of excision", namely the fact that only the individuals that will have lost it, multiply. This allows the use of elimination strategies of a fragment of undesirable exogenous DNA, having a low level of efficiency and, thus, the extension of the fields of investigation in this respect.

In accordance with the invention, the AMS gene is genetically connected to a fragment of undesirable DNA so that the said AMS gene and the said fragment of undesirable DNA can be cut away simultaneously.

The said Fragment of undesirable exogenous DNA can in particular be a marker gene, preferably a gene imparting a resistance to an antibiotic.

The systems of elimination of undesirable genes are generally based on two components: a fragment of excisable DNA that contains the undesirable gene and an inductor of this excision.

According to a method of production of the invention, gene imparting artificial male sterility is introduced in the excisable fragment. The plants transformed with this first component will therefore be sterile males and will be maintained by means of "back - cross - fertilization". The inductor obtained by means of a cross - fertilization, will entail the elimination of the DNA fragment containing the AMS gene, thus causing the development of fruits stemming from self-fertilization. These fruits contain individuals that are relieved from the AMS gene and an undesirable gene.

In practice, it is enough to collect the single seeds produced by means of self-fertilization by F1 plants containing the two components.

According to another method of production of the invention, the plant having integrated into the fragment of excisable DNA containing the AMS gene, can be transformed by means of a fragment of DNA inductor of the excision.

The excision system used in accordance with the invention in order to eliminate the undesirable DNA from the fragment can be a system of transposition, such as in particular the Ac/Ds system of the corn or a system of recombination, such as in particular the Cre/lox system of the bacteriophage P1, the FLP/FRT system of yeast, the recombinase Gin of the phage Mu, the Pine recombinase of E. coli or the system RUR / RS of the pSR1 plasmid.

It is also a goal of the present invention to supply a vector, in particular plasmid, characterized by the fact that it has a fragment of excisable DNA that comprises a fragment of undesirable DNA and the said AMS gene, the said fragment of undesirable DNA being preferably a gene marker, preferably a gene imparting a resistance to an antibiotic, the said AMS gene and the said fragment of undesirable DNA each respectively being linked to elements allowing their expression in the vegetable cellules, especially a promoter and a transcription terminator.

The said vectors according to the invention are used for the transformation of vegetable cellules.

The invention finally aims at a kit for the implementation of an elimination process of a excisable fragment characterized by the fact that on the one hand a vector, as is previously defined, containing a transgene of interest associated to elements allowing its expression in the vegetable cellules, genetically connected to an AMS gene linked with elements allowing its expression in the vegetable cellules, containing an excisable AMS gene or a plant or part of a plant transformed by the said vector, and on the other hand, a vector containing an origin of transposase or recombinase, or a plant or part of a plant transformed by the said vector.

The transformation of vegetable cellules can be realized by means of a transfer of the above-mentioned vector in the protoplasts, especially after incubation of the latter in a solution of polyethylene glycol (PG) in the presence of divalent cations (Ca2+) according to the method described in the article from Krens and al, 1982.

The transformation of the vegetable cellules can also be realized by means of electroporation, especially according to the described method in the article of Fromm and al., 1986.

The transformation of the vegetable cellules can also be realized with the use of a gene cannon allowing, at very great speed, the projection of metallic particles covered with the sequences of the DNA of interest, thus especially delivering genes inside the cellular nucleus according to the described technique in the article of Sanford, (1988).

Another method of transformation of the vegetable cellules, is the one that is called cytoplasmic or nuclear micro - injection.

According to a particularly preferred production method of the process of the invention, the vegetable cellules are transformed by means of a vector according to the invention, by which the said cellular host is susceptible to contaminate the said vegetable cellules allowing the integration of the latter ones into the genome, of the DNA sequences of advantage initially contained in the genome of the above-mentioned vector.

Favorably, the above-mentioned used cellular host is agrobacterium tumefaciens, especially according to the methods described in the articles from Bevan, 1984 and An and al., 1986, or also agrobacterium rhizogenes, especially according to the described method in the article of Jouanin étal., 1987.

In a preferred way, the transformation of the vegetable cellules is realized by means of the transfer of the T region of the circular plasmid, extra-chromosomic inductor of Ti tumors of agrobacterium tumefaciens, using a binary system (Watson and al.).

In order to do so, two vectors are constructed. In one of these vectors, the region of DNA-T was eliminated by means of deletion, with the exception of the upright and left sides, by which a gene marker was inserted between them in order to allow the selection in the plants' cellules. The other partner of the binary system is an auxiliary Ti plasmid, a modified plasmid that does not have DNA-T anymore, but still contains the virulence genes vir, necessary for the transformation of the vegetable cell. The plasmid is kept in agrobacterium.

The AMS gene and the gene of interest can, both or each respectively, be linked with a transcription system of control, especially a promoter and a transcription terminator.

The AMS gene can be associated with a transcription system of control containing a promoter allowing a specific expression in the anther such as the promoter A3 or A9 (WO92 11379) or the promoters TA29, TA26 or TA13 (WO 89 10396).

Among the transcription terminators that can be used, one can cite the terminator polyA35S of the virus of the mosaic of the cauliflower (CaMV), described in the article of Franck and al., (1980), or the terminator polyA NOS which corresponds to the region in 3' non coding of the gene of the synthase nopaline of the Ti plasmid of agrobacterium tumefaciens root in nopaline (Depicker and al., 1982).

Among the transcription promoters that can be used, one can in particular cite, for instance in association with the gene of interest :

- the 35S promoter or favorably the double constitutive promoter 35S (pd35S) of the CaMV, described in the article of Kay and al., 1987 ;

- the PCRU promoter of the gene of the cruciferine of radish allowing the expression of recombinant polypeptides of the invention, only in seeds of the plant obtained from cellules transformed according to the invention in and described in the article of Depigny-This and al., 1992 ;

- the promoters PGEA1 and PGEA6 corresponding with your region 5' non coding of genes of the protein of reserve of seeds, GEAI and GEA6, respectively, of arabidopsis thaliana (Gaubier and al, 1993) and allowing a specific expression in the seeds ;

- the visionary promoter super - promoter PSP (Ni M and al., 1995), constituted of the fusion of a treble repetition of a transcription activator element of the promoter of the gene of the synthase octopine of agrobacterium tumefaciens, of a transcription activator element of the promoter of the gene of mannopine synthase and the promoter mannopine synthase of agrobacterium tumefaciens ;

- the promoter actine of the rice followed by the actine intron of rice (PAR-IAR) contained in the pAct1-F4 plasmid described by Me Elroy and al., 1991 ;

- the promoter HMGW (High Molecular Weight Glutenine) of barley (Andersen O.D. and al., 1989) ;

- the promoter of the gene of yzeine of corn (Pyzeine) contained in the described py63 plasmid in Reina and al. 1990, and allowing the expression in the albumen of the seeds of corn.

Favorably, the gene of male sterility and the gene of interest are linked with one or several sequences coding for a peptide responsible for the addressing of recombinant polypeptides in a certain compartment of the vegetable cell, especially, especially in the endoplasmic reticulum or in the vacuoles or even outside of the cell, in the pectocellulosic partition or in extra - cellular space also called apoplasm.

These sequences coding for a peptide of addressing can be of vegetable, human or animal origin.

Among the sequences coding for a peptide of addressing of vegetable origin, one can cite :

- the nucleotidic sequence of 69 nucleotide (indicated in the examples below) coding for the prepeptide (peptide signal) of 23 amino acids of the sporamine A with soft the potato, by which this peptide signal allows the entry of recombinant polypeptides of the invention in the system of secretion of the vegetable cellules transformed according to the invention (namely mainly in the endoplasmic reticulum) ;

- the nucleotidic sequence of 42 nucleotides (indicated in the examples below) coding for the N-terminal propeptide of vacuolar addressing of 14 amino acids of the sporamine with the soft potato, by which the accumulation of the cellules of recombinant polypeptides of the invention is allowed in the vacuoles of vegetable cellules transformed according to the invention;

- the nucleotidic sequence of 111 nucleotides (indicated in the examples below) coding for the prepropeptide of 37 amino acids of the sporamine A constituted of the N-terminal part towards the C-terminal part of the 23 amino acids of the above-mentioned signal peptide followed by 14 amino acids of the above-mentioned propeptide, by which this prepropeptide allows the entry of recombinant polypeptides of the invention in the system of secretion and their accumulation in vacuoles of the vegetable cellules transformed according to the invention ;

- the three above-mentioned sequences being described in the articles of Murakami and al., 1986 and Matsuoka and al., 1991 ;

- The carboxyterminal propeptide of the lectine of barley in particular described in the articles of Schroeder and al., 1993 and Bednarek and al., 1991 ;

- and the PRS (Pathogenesis Related Protein, Cornelissen and al., 1986) allowing the secretion.

One can also cite among the sequences coding for a peptide of addressing, those coding for the peptides KDEL, SEKDEL and HDEL, in extreme cases C-terminal and allowing an addressing in the endoplasmic reticulum.

Among the vegetable cellules susceptible to be de transformed in accordance with the present invention, one can cite those of the colza, tobacco, corn, pea, tomato, carrot, wheat, barley, potato, soy, litmus , lettuce, rice and lucerne.

The gene of interest to be integrated into the genome of the vegetable cell can in particular be genes coding for proteins of human or animal origin, which can prove to possess a therapeutic or prophylactic advantage, such as the collagen, the gastric lipase, and so on.

The used marker genes can in particular be genes imparting a resistance to antibiotics such as the hygromycine, the canamycine, the bleomycine or the streptomycin or to such herbicides such as the glufosinate, the glyphosate or the bromoxynil.

EXAMPLES

The structure of different plasmids as well as their ligation and the transformation of the escherichia coli DH5a bacteria preliminarily rendered competent, is realized thanks to the usual techniques of recombinant DNA (Sambrook and al., 1989).

EXAMPLE 1

The structure of a binary plasmid associating male sterility imparted by the gene coding the PR-glucanase, the production of the gastric lipase of dog, the selection on canamycine and the selection on basta, usable in the transgenesis of the colza.

The " binary " plasmid derived from pGA492 (An, 1986) that contains between the upright and left sides; derived of the pT'iT37 plasmid of agrobacterium tumefaciens, on its DNA of transfer, the following sequences : the constitutive promoter of the gene nos coding for the synthase nopaline (Depicker and al., 1982), the sequence coding of the NPTII gene for the phosphotransferase neomycine imparting the resistance to the canamycine (Berg and Berg, 1983) deleted of the region of the 8 first codons by which the innovative codon methionine ATG is merged on the sequence of the 14 first codons of the coding sequence of the gene nos (Depicker and al., 1982), by which the coding sequence of the gene nos is deprived of the region of the 14 first codons, the terminator nos (Depicker and al., 1982), a region containing multiple sites of clonage (also called polylinker) (-XbaI-SacI-HpaI-KpnI-ClaI-BglII) preceding the CAT gene coding for the acetyltransferase chloramphenicol (close and Rodriguez, 1982) and the terminating sequences of the gene 6 of the pTiA6 plasmid of agrobacterium tumefaciens (Liu and al., 1993).

a) Structure of the plasmid pBIOC500 bearing the gene imparting male sterility.

The visionary gene corresponding with the "A9-PR-glucanase-T35S promoter" contained in pDW80PR (Worall and al., 1992) is used. The KpnI-EcoRV fragment bearing this visionary gene has been isolated by means of a double enzymatic digestion by KpnI and EcoRV, purified by means of electroelution after electrophoretic migration on gel of agarose at 0,8%, precipitated on alcohol, dried. Il was inserted in the sites KpnI and SmaI of the plasmid pBluescript KS+ marketed by Stratagene. The ligation was realized with 100 ng of the dephosphoryle plasmid and of 50 ng of KpnI-EcoRV fragments in an aggressive environment of 10 µl in the presence of 1 µl of buffer T4 DNA ligase x 10 (Amersham) and of 2.5 U of enzyme T4 DNA ligase (Amersham) at 14°C during 16 hours. The bacteria escherichia coli DH5a, which previously proved to be competent, were transformed. The plasmidic DNA of the obtained clones, selected out of an environment containing 50 µg / milliliter of ampicilline, has been extracted according to the method of the alkaline lyses and analyzed by means of enzymatic digestion by enzymes of restriction. From the obtained plasmid, the fragment KpnI - SstI bearing the visionary gene described above has been introduced in the sites KpnI and SstI of pGA492. The isolation of the visionary fragment is realized by means of the usual process. The ligation was realized with 100 ng of the dephosphoryle vector and of 50 ng of fragment bearing the KpnI-SstI fragment in an aggressive environment of 10 milliliters in the presence of 1 µl of buffer T4 DNA ligase x 10 (Amersham) and of 2.5 U of enzyme T4 DNA ligase (Amersham) at 14°C during 16 hours. The bacteria eschenchia coli DH5a, which previously proved to be competent, were transformed. The plasmidic DNA of the obtained clones, selected out of the environment containing 12 pg / milliliter of tetracycline, has been extracted according to the method of the alkaline lyses and analyzed by means of enzymatic digestion by enzymes of restriction. The obtained vector is called pBIOC500.

b) Structure of the DBIOC501 plasmid bearing the gene imparting the resistance to the Basta.

The EcoRI fragment - HindIII bearing the visionary gene "P35S hyphen PAT- TNOS" isolated from the plasmid pIB16.1 (Broer and al., 1988), is inserted in the sites EcoRI and Hindill of pBSIISK+ marketed by Strategene and modified by means of an addition of a KpnI site in the SmaI site of the polylinker sequence of pBSIISK+. The resulting plasmid is called pBIOC501. The plasmidic DNA of the obtained clones, selected out of an environment containing 50 pg / MT of ampicilline, has been extracted according to the method of the alkaline lysis and analyzed by means of enzymatic digestion by enzymes of restriction.

c) Structure of the DBIOC502 plasmid bearing the coding gene for the gastric lipase from dog.

The gene visionary corresponding with "PCRU-PSLGL-LGC - T35S" isolated from pBIOC93 described in claim WO 9633277, is borne by the fragment obtained by means of a double digestion SacI and XhoI, by which the SacI site is repaired by the effect of the enzyme T4 DNA polymerase (New England Biolabs) according to the recommendations from the manufacturer. This fragment was inserted between the site ApaI influenced by the effect of the enzyme T4 DNA polymerase and the site XhoI of the plasmid pBIOC501. The plasmid resulting is called pBIOC502. The plasmidic DNA of the obtained clones, selected out of the environment containing 50 ug / milliliter of ampicilline, has been extracted according to the method of the alkaline lyses and analyzed by means of enzymatic digestion by enzymes of restriction.

d) Structure of the binary vector pBIOC503.

From pBIOC502, a KpnI fragment bearing the cassettes of expression, "PCRU-PSLGL-LGC-T35S" and "P35S - PAT - TNOS", has been isolated and united in the site KpnI of pBIOC500. The plasmid resulting is called pBIOC503. The plasmidic DNA of the obtained clones, selected out of the environment containing 12 µg / milliliter of ampicilline, has been extracted according to the method of the alkaline lyses and analyzed by means of enzymatic digestion by enzymes of restriction.

The plasmidic DNA of the pBIOC503 plasmid was introduced by means of direct transformation in the root LBA4404 of agrobacterium tumefaciens according to the process of Holsters and al. (1978).

EXAMPLE 2

The structure of a binary plasmid associating male sterility imparted by the gene coding for the barnase, the production of the gastriclipase of dog, the selection on kanamycine and the selection on basta, usable in the trangenesis of the colza.

a) Structure of the plasmid pBIOC504 bearing the gene imparting male sterility.

The visionary gene corresponding with the "promoter A9 barnase - T35S" contained in pWP173 (Paul and al., 1992) has been used. The KpnI - EcoRV fragment bearing this visionary gene has been inserted in the sites KpnI and SmaI of the pBluescript KS+ plasmid marketed by Strategene. From the obtained plasmid, the KpnI - SstI fragment bearing the visionary gene described above has been introduced in the sites KpnI and SstI of pGA492. The obtained vector is called pBIOC504. The plasmidic DNA of the obtained clones, selected out of the environment containing 50 ug / milliliter of ampicillin, has been extracted according to the method of the alkaline lyses and analyzed by means of enzymatic digestion by enzymes of restriction.

b) Structure of the DBIOC501 plasmid bearing the gene imparting resistance toBasta. The vector has been constructed such as described in the example 1 b).

c) Structure of the pBIOC502 plasmid bearing the gene coding for the gastric lipase from dog.

The pBIOC502 vector has been constructed such as described in the example 1 c).

d) Structure of the pBIOC505 vector.

From pBIOC502, a KpnI fragment bearing the cassettes of expression, "PCRU-PSLGL-LGC-T35S" and "P35S - PAT - TNOS", has been isolated and the plasmidic DNA of the obtained clones, selected out of the environment containing 50 pg / milliliter of ampicillin, has been been extracted according to the method of the alkaline lyses and analyzed by means of enzymatic digestion by enzymes of restriction.

The plasmidic DNA of the pBIOC505 plasmid was introduced by means of direct transformation in the root LBA4404 of agrobacterium tumefaciens according to process of Holsters and al. (1978).

EXAMPLE 3

Obtaining of plants f transgenetic colza.

The seeds of colza of spring (Brassica napus CV WESTAR or the lined Limagrain) are disinfected during 40 minutes in a solution of Domestos at 15 %. After four rinses by means of sterile water, the seeds are left to germinate, at the rate of 20 seeds by pot with a diameter of 7 cm and a height of 10 cm from in a mineral environment of Murashige and Skoog (Sigma m 5519) with 30 g / l of saccharose and solidified with Agar gel at 5 g / line. These pots are placed in a culture room at a temperature of 26°C with a photo period of 16H/8H and under a luminous intensity of the order of 80 µE. m-2. S-1.

After five days of germination, the cotyledons are taken in a sterile way by cutting each petiole about 1 mm beyond the knot of cotyledonar.

In a parallel way, a preculture of agrobacterium tumefaciens root LBA4404, containing the binary plasmids, is realized in Erlenmeyer of 50 milliliters, during 36 hours under a temperature of 28°C in a bacterial environment of 10 milliliters 2YT (Sambrook and al., 1989) complemented with antibiotics useful for the selection of the used root.

The preculture serves to inoculate at 1 % a new bacterial culture carried out under the same conditions. At the end of 14 hours, the culture is centrifuged during 15 minutes at 3 000 g and the bacteria are taken back in an equivalent volume of the environment of liquid germination. This suspension is distributed in boxes of Pétri with a diameter of 5 cm and at a rate of 5 milliliter / box.

The edge split of the petiole is submerged in the solution of thus prepared agrobacteria during a few seconds, then, for a few millimeters, the petiole is driven into the environment of regeneration. This environment has the same composition as the environment of germination with moreover 4 mg / l of benzyl-amino-purine (BAP), phytohormone favoring the neoformation of buds. Ten tissue cultures (cotyledon with petiole), are put in a culture by means of a box of Pétri with a diameter of 9 cm (Greiner reference 664102).

After two days of co-culture under the same environmental conditions as the germinations, thee tissue cultures are transplanted in phytatray boxes containing the previous environment (Sigma, reference P1552) complemented with a selective agent : 45 mg / l of sulfate of canamycine (Sigma, reference K 4000) and a bacteriostatic : a mix of 1/6 in weight of salt of potassium of clavulanic acid and of 5/6 set of sodium of amoxicillin (injectable Augmentin(r)) at the rate of 600 mg / line.

Two consecutive times, with an interval of three weeks, the tissue cultures are transplanted in a sterile way into the new environment and under the same conditions.

The green buds that appeared at the end of the second or the third transplantation are separated of the tissue culture and are individually put in a culture in transparent pots with a diameter of 5 cm and a height of 10 cm containing an identical environment as the one before, but deprived of BAP. After three weeks of culture the stem of the transformed bud is split and the bud is transplanted in a pot with a cool environment. At the end of three or four weeks, the roots are developed enough in order to allow the acclimatization of the germ in a phytotron. The buds that are not green or rooted, are eliminated. The germs are then transplanted in bowls of 7 cm at the side filled with mould (norm NF U44551 : 40 % of brown peat, 30 % of sifted heather and 30 % of sand) saturated in water. After two weeks of acclimatization in phytotron (temperature : 21 °C ; photo period :16H/8H and 84 % of relative humidity), the germs are transplanted in pots with a diameter of 12 cm and filled with the same mould enriched in retard fertilizer (Osmocote, at the rate of 4 g / l per mould) and are then transported to the greenhouse (S2 class) under a temperature of 18°C with daily sprayings with water during two minutes.

From the moment of the appearance of flowers, these are put in sacs (Crispac, reference SM 570y 300 mm*700 mm) so as to prevent crossed fertilization.

When the siliques reach maturity, they are gathered , dried and then beaten. The obtained seeds serve to proportioning the biochemical activity of the gene of interest.

The selection of the transgenetic lineage is done by means of germination in an environment of sulfate of canamycine at the rate of 100 to 150 mg / l (according to the genotypes). The operative conditions are identical to the ones described above, as the germinations are carried out in tube of glass with only one seed

per tube. Only the germs developing secondary roots during the first three weeks, are acclimatized in phytotron before they are being passed to the greenhouse.

EXAMPLE 4

Obtaining of plants of transgenetic tobacco.

Tobacco plants used for the transformation experiences {Nicotiana tabacum Var. Xanthi NC and PBD6) are cultivated in vitro in the basic environment of Murashige and Skoog (1962) added up with the vitamins of Gamborg and al (1968), Sigma reference M0404), saccharose at 20 g / l and agar (Merck) at 8 g / l. The pH of the environment is adjusted at 5,8 with a solution of potassium before an autoclave treatment at a temperature of 120°C during 20 minutes. The tobacco germs are transplanted by means of sprouts of the parts to this environment of MS20 multiplication every 30 days.

All the in vitro culture are realized in air-conditioned enclosure, under the conditions defined below:

- luminous intensity of 30 µE.m-2.S-1, photo period of 16 hours;

- thermo-period of 260° C during the day, 24° C during the night. The technique of used transformation is derived from the one of Horsch and al. (1985).

A preculture of the agrobacterium tumefaciens root LBA4404 containing the binary plasmids is realized during 48 hours at a temperature of 28°C under commotion in the LB environment (Sambrook and al., 1989) added up with the appropriate antibiotics (canamycine). Then the preculture is diluted at 1/50 in the same environment and is cultivated under the same conditions. After one night the culture is centrifuged (10 minutes, 3 000 g), the bacteria are resumed in an equivalent volume of liquid MS30 environment (30 g / l saccharose) and this suspension is diluted at 1 / 10.

The tissue cultures of about 1 cm2 are cut up from sheets of germs described above. Then, during one hour, they are put in connection with the bacterial suspension, then they are rapidly dried on filter paper and placed in an environment of co-culture (solid MS30).

After two days the tissue cultures are transferred to boxes of Pétri in the MS30 environment of regeneration, containing a selective agent, the canamycine
(2 000 mg / l), a bacteriostatic, the Augmentin(R) (400 mg / l) and the hormones necessary for the induction of buds (BAP, 1 mg/l and ANA, 0.1 mg / l). A transplantation of the tissue cultures is carried out in the same environment after two weeks of culture. After two more additional weeks, the buds are transplanted in boxes of Pétri in the environment of development composed of the MS20 environment added with canamycine and Augmentin. After fifteen days, half of the buds are transplanted. The rooting takes about 20 days, during which the germs can be given under the form of sprouts of parts or are transferred to the greenhouse.

EXAMPLE 5

Structure of a source of transposase.

A binary plasmid was constructed bearing an origin of transposase fixed under the 35S promoter, because it appeared to be the bearer of a clear and precocious expression of this transposase (Finnegan and al., 1993).

In order to do so, the BamHI-EcoRI fragment of Ac pB135S (built by Finnegan) has been cloned on the sites BamHI and SnaBI of pB1121 (Jefferson and al., 1987). The resulting plasmid called pBIOS144 contains, between the borders on the right and left side, the gene of resistance to the canamycine NPTII under the promoter and terminator nos. The element Ac deleted of its part 5' under the 35S promoter, as such building up the origin of fixed transposase, and 1400 basic pairs of the part 5' of the gene coding for the SS-glucuronidase of E. coli (GUS gene in illustration 3) followed by the nos terminator.

EXAMPLE 6

Structure of the Ds element : KanaR-AMS.

The successive stages having led to manufacturing this vector are the following :

Construction of the plasmid pBIOC203 bearing the gene imparting the resistance to the canamycine.

We inserted the fragment BamHI of 1 Kilobyte (corresponding with the NPTII gene) of pCamVNEO (Fromm and al., 1986) in the BamHI site of pBIOS1 (Perez and al., 1989). The resulting pBIOS vector 1K has been digested by EcoRI. The EcoRI fragment of BIOS 1K has been considered to be appropriate by the polymerase Klenow, then clones in the plasmid AF 3'Ac (Cocherel and al., 1996) in the EcoRI site and thus constituting pBIOS203.

b) Building of the DBIOS208 plasmid bearing the AMS gene.

the EcoRI fragment of the vector pBGS phléo containing 399 basic pairs of the extremity 5' of the element ET Ac (Cocherel al., 1996) has been cloned in pBSsk (Stratagene) in the site EcoRI and thus constituting pBIOS204.

The fragment EcoRI - HindIII (containing the extremity 3 'of Ac and the cassette NPTII) of pBIOS203 has been cloned in pBSsk in the sites EcoRI – HindIII and thus constituting pBIOS205.

The SmaI fragment of pBIOS204 (containing the extremity 5 'of Ac) has been cloned in the SmaI site of pBIOS205 and thus constituting pBIOS206 in.

The SphI fragment of DW 80 bin PR-glucanase containing the gene PR-glucanase (PR-Glu) under the promoter A9 and terminator CaMV (Worall and al., 1992) has been cloned in the site EcoRI of pBIOS206. In the plasmid resulting pBIOS208, the gene A9-PR-glucanase is inserted in the opposite direction in the gene NPTII.

c) Insertion of the element Ds::KanaR-AMS in a binary vector.

The pGA 492DL vector has been obtained after the following modifications of pGA 492 (G. An) :

- deletion of the SacII-ClaI fragment corresponding with the cassette of expression of the visionary NPTII gene. This new vector is called pGa 492D ;

- replacement of the fragment BglII-ScaI (loss of a part of the CAT gene) of pGA 492D by the SacI-KpnI polylinker of pBSIIsk. This new vector is called pGA 492 DL.

The HindIII-SpeI fragment of pBIOS208 has been cloned in the HindIII-SpeI sites of pGA 492 DL in order to constitute the binary pBIOS232 plasmid. The structure of this DNA-T is schematized in the figures 1 and 2.

EXAMPLE 7

Transformation and selection of the tomato.

a) Transformation of the tomato (UC82B variety) built by the binary vector.

The binary pBIOS232 vector has been transferred to the root of agrobacterium LBA 4404, by means of triparental conjugation according to the described technique by Ditta and al.

The transformation of the tomato has been carried out according to the modified technique of J. Fillatti :

Seeds of the UC82B cultivar are sterilized during 15 minutes in 10 % of Domestos and are three times rinsed in sterile water.

They are then sowed in a pot containing the environment of the culture MSSV / 2 during seven or eight days.

The cotyledons are taken and cut up transversally in three parts. Only the central part is preserved and is put in the culture under a temperature of 26°C and in the light, in a KCMS environment added up with of Acetosyringone.

The bacteria of the root agrobacterium LBA 4404 are put in the culture during one night in the environment LB supplemented with the appropriate selective agent(tetracycline). The next day the culture is centrifuged and the obtained bottom is resumed in the liquid KCMS environment .

The tissue cultures are for about 30 minutes soaked in a solution of diluted agrobacterium at 1/20 in the KCMS environment added up with Acetosyringone. They are then rapidly dried on filter paper.

The tissue cultures are then :

- put back in the same KCMS environment during two days in an obscurity under a temperature of 26°C ;

- washed in the liquid environment 2Z added up with Augmentin (400 mg / l) and dried on filter paper :

- transferred to the solid environment 2Z containing 400 mg / l of Augmentin and 100 mg / l of canamycine ; cultivated in the light at a temperature of 26°C ; and

- then transplanted in the fresh environment 2Z, after fifteen days. The first buds appear three weeks after the co-culture. When they reach a height of about 1 cm, they are separated from the expiant and are transplanted in the Dev environment where they take root within one or two weeks if they are transformed in a proper way.

When they are rooted in a proper way, they are transplanted in the lump Jiffy-7 (by AS Jiffy Products) in the phytotron, where they acclimatize rapidly.

From the moment the rooting system is well developed in "Jiffy", the plants are transplanted in a pot with a diameter of 12 cm and then in a container with a diameter of 30 cm, and cultivated in the greenhouse, under automatic spraying and with nourishing solution, (diluted solution at 5 kg/50 l delivered at 1.6 %).

b) Molecular analysis of the primary transformants containing the DNA-T of pBIOS144 or pBIOS232.

When the transformed plants are developed enough in the greenhouse, leafs are then taken (about 5 g) in order to get out the DNA analyzed by Southern Blot afterwards according to the currently used methods (Sambrook and al.).

The DNA of each plant is digested with the Hindill enzyme. After migration and transfer onto the membrane of nitrocellulose, those are subjected to hybridization with different catheters allowing to check :

- the number of reproductions of the inserted DNA-T,

- whether the DNA-T was integrally inserted,

- the presence or the absence of extra edges.

With regard to these molecular analyses, only the plants having a single entire DNA-T without an extra edge, are selected.

The same protocol was implemented in order to alter cellules of tomato with the pBIOS144 plasmid and in order to select the transformed plants.

EXAMPLE 8

Evaluation of the source of fixed transposase.

Once the origin of transposase has been defined and integrated into the plants, we checked if it was able to activate an element "Ds". For this, the selected plants containing the pBIOS144 plasmid, are crossed with a plant containing a testing element Ds by which the excision restores the activity of the Gus gene (p-glucuronidase). The Ds was built in two stages:

- is the BglII-SpeI fragment of pBIOS206 corresponding with the Ds::KanaR has been inserted onto the BamHI-XbaI sites of pB1221(Clontech) in order to obtain pBIOS226.

Then the entire plasmid pBIOS226was inserted in the Hindill site in pGA 492 DL. The resulting binary plasmid pBIOS228 included between the upright and left borders the Ds::KanaR between the 35S promoter and the coding part of the Gus gene.

Any excision of this Ds::KanaR will be translated by an expression of the recorder gene Gus. A test Gus (Jefferson and al.,) has been realized on the F1 descendants of this type of crossing in order to assess the efficiency of the different lines containing the transposase. Blue stains have been detected on cotyledons and sectors on leafs of plants stemming from the crossing between a "transposase" lineage and a "testor Ds" lineage. The number of stains and sectors on a plant are proof of the efficiency from the source of transposase in this plant.

EXAMPLE 9

Generation of lines of defined transposase

The unicopy transformants and those without an extra edge that are selected and positively assessed according to the previous example, were submitted in two successive cycles of self-fertilization. The units of T2 seeds were harvested separately.

For each T2, the segregation was assessed with regard to the resistance to the canamycine in order to detect the units of homozygotic seeds. For each unit about thirty seeds are sowed on earth in the greenhouse. eighteen days after the sowing, a solution of 400 mg / l of canamycine is pulverized on the germs during three days (Weide and al.,). It is easy to identify the plants sensitive to the canamycine after five days. After the segregation of the resistant and sensitive plants and in accordance with Mendelienne segregation of a dominant gene, the units of homozygotic plants are identified for the transgene. Those are the ones that are preferably kept and that will serve to cross the plants containing the Ds::KanaR - AMS element.

EXAMPLE 10

Crossing of the line Ds::KanaR -AMS with the line homozygotic "transposase"

The primary selected transformants (unicopy without extra edge) are assessed from the moment of their blooming in the greenhouse, with regard to the fertility by means of microcospic observation of the transversal cut of the tube of anther of one or two flowers per plant, in carmine vinegar. The outcome of this evaluation is that only the sterile male plants that do not present pollen are preserved.

The sterile plants carrying the Ds:KanaR - AMS are fertilized with pollen of expressing the transposase of Ac. The fruits are harvested and the seeds are extracted.

EXAMPLE 11

A - Identification of the outcome of excision on the F1 plants.

The seeds stemming of the crossing between the plants bearing the source of transposase and those carrying the Ds::KanaR - AMS are sowed in a mould in the greenhouse. At the cotyledons stage, one cotyledon per germ is taken aiming at a rapid extraction of DNA (Lassner and al., 1989) for a PCR test with the oligonucleotides A9a and A9b (Fig. 1). The conditions of the used PCR are the following :

DNA :	40 ng (or 10 pi of the micro-extraction)
oligo A9a (10 pM/µl) :	3 µl
OligoA9b(10pM/µD :	3µl
X10 pad{plug/buffer} :	10 µl
DNTP mix (5 mM) :	4 µl
Taq Promega :	0,4 µl
MgC2 25 mM :	8 µl
H2O up qsp 100 µl	

The reactions are carried out in the Machine Perkin 9600. After 2 minutes of denaturalization at a temperature of 95°C, 40 cycles of the following operations are realized :

Denaturalization 30"de at 94°C
30" of hybridization at 55°C
1'30" of elongation at 72°C

It is the aim of this test to rapidly separate the bearer plants of the AMS gene (line on 800 basic pairs in PCR) of those that does not carry it (no line in PCR). The bearer plants of the AMS gene are transplanted

and during their blooming, the appearance of fruits that would reveal one of the sectors of somatic excision is supervised. The observation of fruits on F1 plants reveals either an excision without reinsertion of the Ds or an excision followed by a reinsertion with a loss of activity.

In order to check this, the seeds stemming from these fruits are extracted and sowed. A molecular analysis by PCR of these plants is realized in order to check that they do not carry the Ds::KanaR - AMS anymore, but that they have the "scar" of the DNA-T and/or the gene of interest. For this, a cotyledon of each germ is taken for the rapid extraction of DNA.

An aliquot of this DNA is taken in order to be subjected to two PCR reactions :

- one with the oligonucleotides EM1 and EM5 (Fig. 2). The Conditions of used PCR are Le following :

DNA :	40 ng (or 10 pi of the micro-extraction)
oligo EM1 (10 pM/µD :	3 µl
oligo EM5 (10 pM/µl) :	3 µl
Pad{Plug/Buffer} times 10 :	10 µl
DNTP mix (5 mM) :	4 µl
Taq Promega :	0,4 µl
MgCl2 25 mM :	18 µl
BSA :	8 µl
Glycerol :	2,5 µl
H2O up qsp 100 µl	

The reactions are carried out in the Machine Perkin 9600. After 2 minute of denaturalization at 95°C, 40 cycles of the following operations are realized :

Denaturalization 30"de in 94°C
30" of hybridization in 62°C
45" of elongation in 72°C

This test allows you to see whether the germ is bearer of the scar of the DNA-T and/or of the gene of interest. In the case the Ds element is cut away, a fragment of about 300 basic pairs is expected, for the scar only, which has to added up to the dimension of the gene of interest;

- the other with the oligonucleotides 5'H and Ac12 (Fig. 3). The conditions of used PCR are the following :

DNA :	40 ng (or 10 pi of the micro-extraction)
oligo 5'H (10 pM/µl) :	3 µl
oligo Ac12(10pM/µl) :	3µl
Buffer x 10 :	10 µl
DNTP mix (5 mM) :	4 µl
Taq Promega :	0,4 µl
MgCl2 25mM :	18 µl
BSA x 100 :	8 µl
Glycerol :	2,5 µl
H2O up qsp 100 µl	

The reactions are carried out in the Machine Perkin 9600. After 2 minute of denaturalization at 95°C, 40 cycles of the following operations are realized :

Denaturalization 30"de in 94°C
30" of hybridization in 62°C
1'30" of elongation in 72°C

This test allows you to check the absence of the coding gene for the transposase. If there is no amplified fragment, the transposase is absent. If there is an amplified fragment, a line of 1.6 Kbyte is detected.

The plants stemming from events of excision, which only carry the gene of interest without the resistance to the canamycine nor the DNA-T bearing the coding gene for the transposase, will be positive with the first exercise of oligonucleotides (EM1-EM5) and negative with the second (5'H-Ac12).

An Southern analysis realized on these plants allows to confirm the presence of the advantageous gene and the absence of the gene of resistance to canamycine.

B - Identification of transformants deprived of the selective marker

1) Equipment :

NPTII catheter :

The amplification of a fragment of 1 KB of the NPTII gene of the pBios232 vector with the oligonucleotides Kana7 and Kana8, of the sequences.

Kana 7 : gctcgacgttgtcactgaag
Kana 8 : cccggaaaacgattccgaag

The used NPTII gene has been isolated from the Tn5 transposon of Escherichia coli (Berg and Berg, 1983). Cat catheters intraLB +intra RB:

Mixture of 2 catheters :

Cat int LB : fragment Sali of the DNA-T of pGA 492 DL containing the left edge and a part of the Cat gene

Int RB ; fragment Sali of the DNA-T of pGA 492 containing the right edge. The whole of the 2 catheters corresponds with the set of the DNA-T of pGA 492 DL that is the basic vector that has served for the construction of the pBios 232 having served to create the plants Ds : KanaR-AMS.

2) Identification and culture of the plants on two active components :

F1 Seeds, stemming from the crossing between a plant carrying the Ds : :KanaR - AMS and a plant bearing the source of defined transposase, have been obtained and have been sowed. A PCR test on the DNA of cotyledons of 173 plants has been done with the oligonucleotides EM1 and EM5 as described before. This test allowed to identify 18 plants by which a line of 350 pb has been amplified by EM1-EM5 and, as such, revealing somatic excision, which is proof of the presence and the functionality of the 2 elements of the system.

The system was finalized with an DNA-T carrying a Ds : :KanaR - AMS but without a gene of interest. Therefore, the line of excision revealed by amplification done with EM1 and EM5 is about 350 pb. In the case by which an advantageous gene is inserted, this line will be lengthened with the dimension of this gene. According to another method of production, one can also use specific oligonucleotides of the gene of interest.

These 18 plants were cultivated in the field during 4 months. The observation of the flowers is regularly done in order to verify the fertility of the anthers as well as the appearance of fruits. With 3 out of 18 plants, fruits only appeared on certain bunches. These plants were therefore visionary for the fertile male / sterile male phenotypes. The fruits on these plants were harvested bunch per bunch while noticing the hierarchy of the bunch in comparison with the whole plant.

Seeds of fruits appeared on 3 bunches of 3 plants that partially became fertile again, were sowed. Leafs of obtained germs (4 germs / bunch), were taken with the aim to an extraction of DNA for an analysis of the Southern type. The DNA is subjected to a digestion with the enzyme Hind III (single site in the DNA-T) before being subjected to an electrophoresis and a transfer to the membrane. The 36 samples corresponding with the F2 descendants were treated at the same time as the parents of the hybrid of the DNA (the plant carrying the Ds : :KanaR-AMS and the plant bearing the source of the defined transposase), in such a way to obtain the reference profiles. The hybridization of the Southern blot with the catheters NPTII and Cat-int LB allowed to identify the plants that did no-more carry the DNA-T containing the source of transposase nor the Ds : :KanaR-AMS, but which contained the scar of the DNA-T, thanks to comparison with the profile of the parents.

We analyzed 36 plants in total, without preliminary selection in the canamycine, deriving of 3 bunches of 3 plants by which fruits were observed.

3) Evaluation of the analysis :

20 plants carrying the DNA-T with the scar, that is revealing 1 line of hybridizing with the catheter Cat int LB-RB, with a dimension inferior to the one present in the parent carrying the Ds. This same line is not hybridized by the NPTII catheter.

31 plants bearing the source of transposase, that is revealing a line of hybridizing with the NPTII catheter, with an identical dimension as is revealed by the same catheter with the parent bearing the source of defined transposase.

5 plants carrying the empty DNA-T, but not bearing the source of transposase.

On 36 analyzed plants, 5 appeared to be proper transformants that did no more carry the bearer DNA-T of the gene of interest and by which the selecting marker gene was cut away.

Therefore it is possible to select 2 generations of the properly transformed plants in accordance with the primary transformant.

The advantages of this technique are multiple. It allows to identify in a precocious way the transformants deprived of the selective marker.

The crossing of the primary transformant with the source of transposase is made easier and more reliable because this one is male sterile.

This technique finally allows the screening of a low number of germs in order to identify the proper transforming : without preliminary pre-selection, after analysis of 36 descendants of 3 plants, 5 plants containing the DNA-T deprived of the Ds : :KanaR- AMS were identified.

Overmore, it is possible to improve the identification of the transformants deprived of markers :

If we preliminarily the descendants of fruits produced on a plant carrying the Ds : :KanaR- AMS and the source of transposase in the canamycine, one should obtain a quarter of the plants KanaR instead of 15/16 (normally obtained if there was no excision of the Ds). Among these, % are bearer of the proper DNA-T, by which the other quarter corresponds with the classical segregants that carry no transgene. Moreover, because of the observation of the segregation of 3/4 of resistant plants for 1/4 of sensitive plants, it is possible to confirm that the system of elimination was effective.

It is therefore possible to identify proper descendants while analyzing very few plants among the sensitive F2 germs in the canamycine.

4) conclusion :

This study shows that the adding of a gene imparting the artificial male sterility on the system of elimination of the marker gene by Ac / Ds, allows to rapidly identify the descendants bearing the gene of interest without the selective marker, while the process is made easier and while preventing the dissemination by pollen of transgenetic events insufficiently characterized.

EXAMPLE 12

Structure of a binary plasmid pBIOC4-FLP carrying the FLP recombinase.

The sequence coding the FLP recombinase of Saccharomyces cerevisiae is included in pOG44 marketed by Stratagene. The pOG44 plasmid was digested by the enzymes Hindill and Kpnl and the HindIII-Kpnl fragment was isolated. The ligation has been realized with 100 ng of the dephosphorylized vector pBIOS512 and 50 ng of the HindIII-Kpnl fragment carrying the sequence coding the recombinase in an aggressive environment of 10 pi in the presence of 1 µl of buffer T4 DNA ligase x 10 (Amersham) and 2.5 U of enzyme T4 DNA ligase (Amersham) at a temperature of 14°C during 16 hours. The bacteria Escherichia coli DH5o(preliminarily considered to be competent}, were transformed. The resulting plasmid is called pBIOS512-FLP. The plasmidic DNA of the obtained clones, selected in the environment containing 50 µg / milliliter of ampicilline, has been extracted according to the method of the alkaline lyses and has been analyzed by means of enzymatic digestion by enzymes of restriction. The fragment bearing the sequence coding the FLP recombinase has been isolated by means of enzymatic digestion by Kpnl followed by the effect of the enzyme T4 DNA Polymerase (New England Biolabs) according to the recommendations of the

manufacturer following the digestion by HindIII. The fragment was purified by electrophoresis on gel of agarose at 0,8%, electro-eluted, precipitated on alcohol and dried. It was introduced in the BamHI site, treated by the Klenow enzyme (New England Biolabs) according to the recommendations of the manufacturer and in the HindIII site of the pBIOS512 plasmid. The nucleotidic sequence of FLP was checked by means of sequencing with the help of the sequencing kit of T7? marketed by Pharmacia according to the method of the didesoxynucleotides (Sanger and al., 1977). The pBIOS512 plasmid derives of pUC and carries the cassette of expression " double promoter 35S (PD35S) - terminator NOS (TNOS)" (fragment EcoRI). The double 35S promoter derives of pJIT163 (WO 96 33277).

From pBIOS512-FLP, the EcoRI fragment carrying the cassette of expression PD35S-FLP-TNOS has been isolated by means of enzymatic digestion by EcoRI, purified on gel of agarose at 0,8%, electro-eluted precipitated on alcohol and dried. It was introduced on the site EcoRI of pBIOC4 (WO 96 33277) dephosphorylized by the alkaline phosphatase enzyme of the cow intestines (Boehringer Mannheim) according to the recommendations of the manufacturer. The ligation has been realized with 100 ng of the dephosphorylized vector and 50 ng of the fragments carrying the cassette PD35S-FLP-TNOS in an aggressive environment of 10 pi in the presence of 1 µl of buffer T4 DNA ligase x 10 (Amersham) and 2.5 U of enzyme T4 DNA ligase (Amersham) at a temperature of 14°C during 16 hours. The bacteria Escherichia coli DH5cc preliminarily considered to be competent, were transformed. The resulting plasmid is called pBIOC4-FLP. The plasmidic DNA of the obtained clones, selected in the environment containing 12 µg / milliliter of tetracycline, has been extracted according to the method of the alkaline lyses and analyzed by means of enzymatic digestion by enzymes of restriction. The plasmidic DNA of the binary pBIOC4-FLP plasmid has been introduced by direct transformation in the root LBA4404 of Agrobacterium tumefaciens according to process of Holsters and al. (1978). The validity of the retained clone was checked by means of enzymatic digestion of the introduced plasmidic DNA.

EXAMPLE 13

Structure of a binary plasmid and pBIOC511, bearing the male sterility and the selection on canamycine amongst sites of specific FRT recombination, usable in the transgenesis of tobacco.

The binary plasmid pGA492 (Year, 1986) was deleted of the following sequences : the constitutive promoter of the nos gene coding for the nopaline synthase (Depicker and al., 1982)., the coding sequence of the nptII gene coding for the neomycine phosphotransferase II (Berg and Berg, 1983) deleted of the region of the 8 first codons by which the initiating codon methionine ATG is merged on the sequence of the 14 first codons of the coding sequence of the nos gene (Depicker and al., 1982), the coding sequence of the nos gene deprived of the region of the first 14 codons, the nos terminator (Depicker and al., 1982). This deletion was realized with total ClaI digestion, followed by a partial SacII digestion. The fragment is purified, then subjected to the effect of the enzyme T4 DNA Polymerase (New England Biolabs) according to the recommendations of the manufacturer. The ligation of the plasmid and the transformation of Escherichia coli DH5a bacteria preliminarily considered to be competent, are realized while following the usual process (Sambrook and al., 1989). The resulting plasmid is called pBIOC506.

The site of specific FRT integration was amplified by PCR from the pOG45 plasmid marketed by Strategene. The two oligodesoxynucléotides used as stimulator for the PCR are :

CGC CTG CAG TTT TCA AAA GCG CTC TGA AGT TC' 3 CGC CTG CAG TTT TCA AAA GCG CTC TGA AGT TC' and

CCA AAG CTT GAA TTC GCC AGG GGG ATC TTG AAG TTC' 3 CCA AAG CTT GAA TTC GCC AGG GGG ATC TTG AAG TTC'.

The corresponding fragments on the FRT site, stemming of PCR amplification have been digested by PstI, submitted to the effect of the enzyme T4 DNA Polymerase (New England Biolabs) according to the recommendations of the manufacturer and are then digested by EcoRI. They were purified on gel of agarose at 2 % , electro-eluted, precipitated on alcohol, dried and put back in water. Then they were united between the SacI site preliminarily submitted to the effect of the enzyme T4 DNA Polymerase and the EcoRI site of the pUC18 plasmid marketed by Pharmacia. The resulting plasmid is called pBIOC507.

A second FRT site was introduced in the pBIOC507 plasmid. This second FRT site is the result of the PCR amplification with the help of the two oligodesoxynucleotides :

CGC CTG CAG TTT TCA AAA GCG CTC TGA AGT TC' 3 CGC CTG CAG TTT TCA AAA GCG CTC TGA AGT TC' and

AAA GGT ACC GCC AGG GGG ATC TTG AAG TTC' 3 AAA GGT ACC GCC AGG GGG ATC TTG AAG TTC'.

The amplified fragments are digested by the Psti and KpnI, are submitted to the effect of the enzyme T4 DNA Polymerase and are united in the Sphi site preliminarily treated by the effect of the enzyme T4 DNA Polymerase, of the pBIOC507 plasmid. The resulting plasmid is called pBIOC508 and contains the two FRT sites positioned in the same direction.

From the pBIOC508 plasmid, the HindIII - EcoRI fragment bearing the two FRT sites, was isolated. This fragment was inserted on the HindIII and EcoRI sites of the pBIOC506 plasmid. The resulting plasmid is called pBIOC509.

In order to impart the male sterility, the Sphi fragment bearing the visionary gene corresponding with the "promoter A9 - barnase -T35S " contained in pWP173 (Paul and al., 1992) has been used. This Sphi fragment was treated by the effect of the enzyme T4 DNA Polymerase and united in the PstI site treated by the effect of the enzyme T4 DNA Polymerase of the pBIOC509 plasmid. The resulting plasmid is called pBIOC510.

Finally, to allow the selection on canamycine (Fromm and al., 1986), the EcoRI fragment treated by the effect of the enzyme T4 DNA Polymerase, corresponding with the cassette of expression "PNOS - nptII - TNOS" isolated from a plasmid of pBIOS1 in which the BamH1 fragmenthas been cloned in the site BamH1 coding for the nptil gene, has been united in the KpnI site treated by the effect of the enzyme T4 DNA Polymerase of the pBIOC510 plasmid. The resulting plasmid is called pBIOC511.

CULTURE ENVIRONMENTS

LB

10 g / l Bactotryptone
5 g / l got out from yeast
10 g / l NaCl
PH 7,5 adjusted with NaOH

MSSV / 2

2.2 macro-instruction g and microphone-elements of Murashige and Skoog (ref M 6899 Sigma)
2 milliliter / l vitamins of Nitsch
15 g / l saccharose
8 g / l Agar-Agar
PH 5,9 before autoclaving

2Z

4.4 macro-instruction g and microphone-elements of Murashige and Skoog (ref M 6899 Sigma)
2 milliliter / l vitamins of Nitsch
30 g / l saccharose
2 Zeatine mg/l
400 Augmentin mg/l
100 canamycine mg/l
8 g / l Agar-Agar
PH 5,9 before autoclaving

KCMS

4.4 macro-instruction g and microphone-elements of Murashige and Skoog (ref M 6899 Sigma)
0.9 Thiamine mg/l
200 mg/l potassium dihydrogenophosphate
30 g / l saccharose
8 g / l Agar-Agar
200 Acetosyringone µM
0.2 mg/l 2-4D
0.1 cinetine mg/l
PH 5,9 before autoclaving

DEV

2.2 macro-instruction g and microphone-elements of Murashige and Skoog (ref M 6899 Sigma)
2 milliliter / l vitamins of Nitsch
15 g / l saccharose
50 canamycine mg/l
400 Augmentin mg/l
8 g / l Agar-Agar
PH 5,9 before autoclaving

MS20

4.4 g / l M0404 (SIGMA)
20 g / l Saccharose
8 g / l Agar-Agar (solid environment)
PH 5,7
+ possibly vegetable hormones BAP 1 mg/l and ANA 0.1 mg / l

MS30

4.4 g / l M0404 (SIGMA)
30 g / l Saccharose
8 g / l Agar-Agar (solid environment)
PH 5,7
+ possibly vegetable hormones BAP 1 mg/l and ANA 0.1 mg / l

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CLAIMS

1. Use of a sterile male plant in order to avoid the dissemination of a transgene of interest integrated into the genome of the said plant.

2. Use according to claim 1 by which the said plant is bearer of male cytoplasmic sterility.

3. Use according to claim 1 by which the said plant is bearer of nuclear male sterility.

4. Use of an excisable AMS - gene as a means allowing the screening of the genetically transformed plants having integrated a transgene of interest and having eliminated a fragment of excisable DNA, by which the said fragment of the excisable DNA can :

   - either be the said AMS - gene,

   - or contain the AMS - gene genetically connected with a fragment

   of undesirable DNA, so that the said AMS - gene and the said fragment of undesirable DNA can simultaneously be cut away get, by which the said fragment of undesirable DNA preferably is a marker gene, preferably a gene imparting a resistance to an antibiotic.

5. Use according to one of the previous claims, characterized by the fact that the said plant fixes is chosen out of the corn, colza, the tobacco or the tomato.

6. A transgenetic plant or an extract or a part of a transgenetic plant, characterized by the fact that the said transgene of interest is genetically linked with an AMS - gene linked with elements allowing its expression in the vegetable cellules, especially a promoter and a transcription terminator.

7. Vector, in particular plasmid, characterized by the fact that it contains a transgene of interest linked with elements allowing its expression in the vegetable cellules, in particular a promoter and a terminator of transcription, genetically connected with an AMS - gene linked to elements allowing its expression in the vegetable cellules, especially a promoter a terminator of transcription.

8. Cellular host, especially bacterium such as Agrobacterium tumefaciens, transformed by a vector according to claim 7.

9. Process of insertion of an AMS - gene in a plant characterized by the fact that it copprises the transformation of vegetable cellules, especially with the help of a cellular host according to claim 8, by which it is transformed by a vector according to claim 7, in such a way that an AMS gene genetically linked with a transgene of interest can be integrated into the genome of its cellules.

10. Process of elimination of a fragment of excisable DNA, characterized by the fact that it comprises:

    - either

      a) The transformation of vegetable cellules, especially with the help of a cellular host according to claim 8, by which it is transformed by a vector according to the claim 7 containing an excisable AMS - gene, in such a way that the said AMS - gene, excisable by recombination or transposition, can be integrated into the genome of its cellules;

      b) the regeneration of plants transformed from the vegetable cellules that are transformed as described above ;

      c) the transformation of vegetable cellules in such a way that an inductor element of transposition or recombination can be integrated into the genome of its cellules;

d) the regeneration of plants transformed from the vegetable cellules that are transformed as is mentioned in step c);

e) the crossing of the sterile male plants obtained in step b) with the lines expressing the transposase or the recombinase obtained in step d), events of excision are done among the F1 plants.

-or

a) the transformation of vegetable cellules, especially with the help of a cellular host according to the claim 8, by which it is transformed by a vector according to the claim 7 containing an excisable AMS

- gene, in such a way that the said AMS - gene excisable by means of recombination or transposition can be integrated into the genome of cellules ;

b) the regeneration of plants transformed from the vegetable cellules that are transformed as described above ;

c) the transformation of the vegetable cellules of the above mentioned transformed plants in such a way that an inductor element of transposition or recombination can be integrated into the genome of its cellules;

d) the regeneration of plants transformed from the vegetable cellules that are transformed as is mentioned in step c).

11. Production process of a product of expression of a transgene of advantage{interest} characterized by the fact that it comprises :

a)

- either the transformation of vegetable cellules, especially with the help of a cellular host according to claim 8, by which it is transformed by a vector according to the claim 7, in such a way that an AMS – gene, that is genetically connected with a transgene of interest, can be integrated in the genome of its cellules;

- or the transformation of vegetable cellules bearing male cytoplasmic or nuclear sterility in such a way to integrate a transgene of interest into the genome of its cellules ;

b) the regeneration of plants transformed from the vegetable cellules that are transformed as is mentioned above ;

c) the recovery of the product of expression of the said transgene of interest in the said cellules or above mentioned transformed plants, in particular by means of extraction and , as the case may be, by means of purification.

12. Kit for the execution of the process according to claim 10 characterized by the fact that on the one hand it contains he a vector according to claim 7, containing an excisable AMS - gene or a plant or a part of a plant transformed by the said vector, and on the other hand a vector bearing the source of transposase or recombinase or a plant or part of a plant transformed by the said vector.